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# **Human eccrine sweat gland cells can reconstitute a stratified epidermis**

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Abbreviations: SwGld, sweat gland; KdES, keratinocyte derived epidermal substitute; SdES, sweat gland derived epidermal substitute; K, (cyto)keratin

## **Abstract**

Eccrine sweat glands are generally considered a possible epidermal stem cell source. Here we compared the multilayered epithelia formed by epidermal keratinocytes and eccrine sweat gland cells respectively. We demonstrate both in vitro and in vivo the capability of human eccrine sweat gland cells to form a stratified interfollicular epidermis substitute on collagen hydrogels. This is substantiated by the following findings: (1) A stratified epidermis consisting of 10 -12 cell layers is formed by sweat gland (SwGld) cells. (2) A distinct stratum corneum develops and is maintained after transplantation onto immuno-incompetent rats. (3) Proteins such as filaggrin, loricrin, involucrin, envoplakin, periplakin, and transglutaminases I and III, are matching the pattern of normal human skin. (4) Junctional complexes and hemidesmosomes are readily and regularly established. (5) Cell proliferation in the basal layer reaches homeostatic levels. (6) The SwGld-derived epidermis is anchored by hemidesmosomes within a well developed basal lamina. (7) Palmo-plantar or mucosal markers are not expressed in the SwGld-derived epidermis.

These data suggest that human eccrine sweat glands are an additional source of keratinocytes that can generate a stratified epidermis. Our findings raise the question as to which extent human skin is repaired and/or permanently renewed by eccrine SwGld cells.

## Introduction

Keratinocyte stem cells are supposed to assure the homeostatic renewal of the epidermis and to be responsible for re-epithelialisation of the epidermis after skin injuries. Keratinocytes stem cells do not only reside in the epidermis but also in appendageal structures protruding into the dermis. Outer root sheath cells of hair follicles were shown to be able to regenerate a fully differentiated epidermis in vitro and were used for the production of epidermal equivalents for the treatment of skin damage or disease (Lenoir et al., 1988; Limat et al., 2003; Limat et al., 1996).

The bulge region of both mouse and human hair follicles has been shown to contain a multipotent stem cell population (reviewed in (Alonso and Fuchs, 2003; Cotsarelis, 2006)). In the mouse these stem cells do not appear to contribute to epidermal regeneration under homeostatic conditions (Ito et al., 2005; Levy et al., 2005). It remains to be investigated whether this is also true for human skin. Interestingly, human glabrous skin, which is completely free of hair follicles, but contains a high number of sweat glands (Frinkel and Woodley, 2001), also exhibits regenerative potential. This fact prompted us to ask whether sweat gland (SwGld) derived epithelial cells can differentiate into keratinocytes and form an epidermis.

Human eccrine sweat ducts develop at around gestational week 15 to 20 by budding from the basal cell layer. The resulting anlagen protrude into the dermis forming the globular secretory domain (Ersch and Stallmach, 1999; Moll and Moll, 1992). Because very little is known about the differentiation of sweat glands, any effort to regenerate them in vitro remains extremely ambitious (Shikiji et al., 2003). There are some indications that sweat gland cells may have a certain capacity to form an epithelium in culture and in vivo (Jones et al., 1988; Lobitz et al., 1954; Miller et al.,

1998). Yet, these few studies did not address the issue of the type and quality of the epithelium produced.

The presence of a sufficient number of viable epidermal stem cells in cultured epidermal autografts is of major clinical importance (Auger et al., 2004; De Luca et al., 2006; MacNeil, 2007; O'Connor et al., 1981). We reasoned that human eccrine sweat glands may be an additional source of epidermal (stem) cells which may contribute to the in vitro maintenance and long term survival and function of engineered epidermal grafts.

To address this issue we raised organotypic cultures consisting of a dermal component and of a stratified epidermal substitute (ES) formed by epidermal keratinocytes or sweat gland cells respectively. These substitutes designated KdES (Keratinocyte derived Epidermal Substitute) and SdES (Sweat gland derived Epidermal Substitute) were transplanted onto the backs of immuno-incompetent rats and the expression of protein markers indicative of the grade of differentiation and homeostasis was determined. Although with a delay in comparison to epidermal keratinocytes, SwGld derived cells developed 'de novo' into a functional stratified, interfollicular epidermis.

## Results

### **Sweat gland derived epithelial cells acquire properties of epidermal keratinocytes in engineered dermo-epidermal substitutes.**

Human eccrine sweat glands were isolated from the scalp, abdomen and the retro-auricular area of young patients ranging in age from 1 to 18 years. As depicted in Fig.1a, preparations of glandular structures consisted of the secretory domain (white arrowheads), the intraglandular duct and stretches of the intradermal duct of varying length (black arrows). Isolated intradermal ducts always lacked the intra-epidermal acrosyringium and a significant stretch of the upper duct (Fig. 1a). Consequently, epidermal cells were avoided in sweat gland-derived cell preparations. Keratinocytes were isolated mainly from foreskins. Both cell types were expanded during passage 1. In Fig.1b the outgrowth of the above mentioned three domains of an eccrine sweat gland is depicted. Myoepithelial cells were associated with secretory SwGld cells and were co-isolated. They were detected by smooth muscle actin expression and showed to disappear from the culture within 5-7 days (data not shown).

In the following, SwGld-derived (3D) cell cultures were always compared to the corresponding cultures of keratinocytes. The cells were initially seeded within a ring of  $0.7\text{cm}^2$ , grown on collagen type I hydrogels that had been previously populated with  $1 \times 10^5$  primary human dermal fibroblasts. Keratinocytes finally covered the entire gel, thereby showing surface expansion of about 4 times (Fig.1c). SwGld cells expanded somewhat slower, increasing the surface of the initial cell layer approximately 2.5 times (Fig.1d).

After the in vitro culture phase the composite substitutes consisting of a dermal and a multilayered epidermal equivalent, were transplanted onto the backs of immuno-incompetent Nu/Nu rats. All following comparative analyses were performed on

histological sections of dermo-epidermal grafts after their transplantation onto immuno-incompetent rats. To prevent ingrowth of rat-derived skin cells, a transplantation chamber was used as previously described (Pontiggia et al., 2009) (Figs. 1e,f). Histological analyses 21 days after transplantation revealed that both, epidermal keratinocytes and SwGld cells formed a stratified epidermis which consisted of a basal layer, 10-12 suprabasal layers and a well differentiated anuclear stratum corneum (Figs. 1g,h).

### **Characteristics of a stratified epidermis formed by SwGld cells**

Due to the microdissection technique employed, it is unlikely that our SwGld cell preparations were contaminated by epidermal keratinocytes. Nevertheless, we performed control experiments to discriminate between keratinocytes and sweat gland cells in the initial cell preparations (Figs 2c,d) and in SdES and KdES (Figs 3a-f). As shown in Tab. 1 only a very limited selection of antibodies, such as antibodies to K2e, K8 could be employed for these analyses. K2e is expressed in all suprabasal keratinocytes of the human epidermis (Smith et al., 1999), whereas it can not be recognized in the sweat gland, not even in the intraepidermal acrosyringium (Figs. 2a,b). As shown in Fig. 2b, K8 expression is confined to the secretory domain of the sweat gland.

Cytospins of newly prepared primary SwGld cells and keratinocytes were analyzed for the expression of K2e and K8. Importantly, none of the SwGld cell preparations (n=3) contained K2e-expressing cells, while about 50% were positive for K8 (Fig. 2d). These findings indicate that the SwGld cell preparations were not contaminated by (suprabasal) epidermal keratinocytes. In contrast the majority of epidermal keratinocytes expressed K2e, whereas these cells did not stain for K8 (Fig. 2c). Although epidermal keratinocytes completely down-regulated K2e expression on cell

culture plastic (data not shown), a significant number of them re-expressed K2e when integrated into differentiated epidermal substitutes, while K8 was not expressed by epidermal keratinocytes (Fig. 2e).

One significant difference between human eccrine SwGld cells and epidermal keratinocytes was, that only a relatively low number of SwGld cells expressed K2e within 5 to 7 weeks in differentiating epidermal equivalents (Fig. 2f). Notably, the number of K2e-expressing cells in SdES did not significantly increase 7 to 10 weeks after their transplantation (data not shown). Thus, the K2e-expressing cells in SdES may represent SwGld-derived cells that underwent additional epidermal differentiation steps. It remains to be investigated, however, whether the number of K2e-positive SwGld cells would still increase during an extended in vivo differentiation period of three or more months. Notably, K8 positive cells were completely absent in SdES, indicating that this (secretory domain-specific) sweat gland marker was no longer expressed in the engineered epidermis (Fig. 2f).

In a next experimental series we set out to explore whether the SdES exhibited mucosal or palmoplantar characteristics. This was done by screening the two types of epidermal substitutes for the expression of K4, a mucosal marker, and K9, a marker of palmoplantar epidermis. In none of these experiments (n=4) we found expression of K4, indicating that there was no mucosal differentiation. Furthermore, neither in KdES nor in SdES, K9-positive cells were detected (n=4), showing that palmoplantar properties were absent (Figs. 2g, h).

To monitor the contribution of rat keratinocytes to the formation of the epidermal substitute, we intended to confirm the human origin of the substitutes. Paraffin sections of engineered transplants were subjected to in situ hybridisation using DNA probes specifically hybridizing to primate-specific Alu-repeats. Fig. 2i shows the well-defined transition zone (black dotted line) between the graft of human origin and the



underlying, unstained rat tissue. Immunostaining for a human-specific nuclear antigen and for the human fibroblast specific CD90/Thy-1 antigen also confirmed the human origin of the analyzed tissue sections (data not shown).

As there was no reliable antibody allowing to discriminate between basal epidermal keratinocytes and sweat gland cells respectively (see also Tab. 1), we employed an indirect assay, determining whether cells of the epidermal stratum basale were “contaminating” the sweat gland cell fraction. This was achieved by screening the two different types of epidermal substitutes (KdES and SdES) for the presence of epidermal melanocytes. Melanocytes are located in the epidermal stratum basale, and are always present in epidermal keratinocyte preparations, whereas they are absent in eccrine sweat glands. We were able to show that melanocytes were present in all KdES investigated (Fig. 3a), whereas no melanocytes could be detected in SdES (Fig. 3b). Accordingly (and not surprisingly) we showed that the transfer of melanin from melanocytes to keratinocytes takes place in KdES (Figs 3c,e), whereas this was not possible in SdES (Figs. 3d,f).

### **Homeostasis of transplanted epidermal substitutes.**

To determine the proliferative capacity of the epithelial cells in the epidermal substitutes, frozen sections were stained for Ki-67, a nuclear cell proliferation-associated antigen, expressed in all active stages of the cell cycle (Gerdes et al., 1991). In unwounded, homeostatic human skin, Ki-67 was expressed in single keratinocytes in the basal and first subbasal layers in the interfollicular epidermis (suppl. Fig. S1a). We observed Ki-67 positive cells in the basal layer of both types of epidermal substitutes (Figs. 4a, b) three weeks after transplantation indicating that the stratification process was ongoing and that, as in normal skin, only a small

subpopulation of keratinocytes maintained epidermal renewal. An identical pattern of proliferative cells was observed in 7 weeks old grafts (Fig. 4c).

Normal, homeostatic interfollicular epidermis lacks the expression of K16 and K17 (Moll et al., 1983; Troyanovsky et al., 1989) (see also Fig. S1a). These keratins are induced in suprabasal, post-mitotic keratinocytes when tissue homeostasis is perturbed in wound repair situations, such as the re-epithelialization after transplantation of dermo-epidermal substitutes or in cultures of dissociated keratinocytes grown on cell culture plastic (Coulombe, 1997; Leigh et al., 1995; Paladini et al., 1996). In both, KdESs and SdESs the expression of K16 was clearly detected in suprabasal cell layers early after transplantation (Figs. 4a, b) indicating a persistent wound healing activity. At about 7 to 10 weeks after transplantation K16 was somewhat down-regulated in both types of substitutes (Fig. 4c), and data not shown).

### **Stratification of engineered epidermal equivalents**

Involucrin, envoplakin and periplakin are markers of the cornification process and are associated with the formation of desmosomes and intermediate filaments (DiColandrea et al., 2000). In normal human skin involucrin and loricrin are expressed in the upper granular and cornified layers (Fig. S1c).

We found that the “early” pattern of involucrin expression differed from that of homeostatic skin in both types of substitutes. In fact, a strong expression was visible in the spinous layer, three weeks after transplantation (Figs. 4d,e). Seven weeks after transplantation, as stratification and tissue homeostasis had further progressed, the lower suprabasal layers had down-regulated involucrin expression (Fig. 4f). The expression pattern of loricrin was similar to that of normal skin, both in the KdESs

and in the SdESs, three weeks (Figs. 4d, e) and 7 weeks (Fig. 4f) after transplantation. Expression of envoplakin, filaggrin and the transglutaminases 1 and 3, essentially confirmed the findings obtained with loricrin and involucrin (data not shown). Once again, the differences between KdESs and SdESs were moderate.

The distribution of K1/10, as late differentiation markers (Patel et al., 2006) is indicative for the degree of tissue homeostasis in normal human skin. In fact, during the stratification process K10 appeared earlier in all suprabasal layers, while K1 expression was delayed and started in more upper layers (Stark et al., 1999). KdES (Fig. 4g) eventually showed a staining pattern comparable to that of normal skin (see also suppl. Fig. S1d). In general, the stratification process of the SdES was delayed in comparison to the KdES, with K1 being expressed after three weeks only in the upper stratum spinosum and granulosum (Fig. 4h). In SdES the expression pattern of normal human skin was reached at about 7 weeks after transplantation (Fig. 4i).

During stratification the epidermis establishes an intricate network of different intercellular junctional complexes to establish its functions as a barrier against external agents, as a water-balance regulator, and as a nutrient circulator (Dusek et al., 2007; Morita and Miyachi, 2003; Perez-Moreno et al., 2003). We analyzed the presence of tight junctions, since together with the lamellar bodies of the cornified envelope, these establish the epidermal diffusion barrier (Malminen et al., 2003; Schluter et al., 2004). One of the major constituents of tight junctions, occludin, is expressed in the upper granular layer of human skin (Fig. S1e). Its typical expression pattern was obtained in both types of ES, 3 weeks after transplantation (Figs. 4k, l). However, in the SdES we observed stronger and more homogeneous occludin expression only at 7 weeks after transplantation (Fig. 4m).

E-cadherin is expressed as a component of adherens junctions in the living keratinocyte layers of the human epidermis (Moles and Watt, 1997) (see also Fig.

S1e). Both types of epidermal substitutes showed the expression pattern of normal epidermis (Figs. 4k, l, m).

### **Desmosomes and the dermo-epidermal junction in engineered SdES**

The desmosomal proteins desmoglein (Dsg) 1 and 3 are expressed in a graded fashion in human epidermis. Dsg3 is expressed in the stratum basale, whereas Dsg1 can be detected in upper layers (Fig. S1f). Dsg 1 and 3 respectively their partners desmocollin 1 and 3 are involved in the regulation of epidermal differentiation (Dusek et al., 2007; Elias et al., 2001). Dsg 3 is expressed exclusively in the ductal portion of the SwGId (data not shown). During the first three weeks after transplantation both types of substitutes showed a somewhat unbalanced (mixed) expression pattern of Dsg1 and Dsg3. The suprabasal layers co-expressed both proteins in comparable quantities (Figs. 5a, b). For the establishment of the physiological protein gradients, longer time periods (7 weeks and more) were necessary (Fig. 5c).

Periplakin was detected in all suprabasal layers, but was more prominent in the upper spinous and granular layers (Figs. 5 d,f and Fig. S1b). Once again, this pattern was not yet completely established in SdES after 3 weeks (Fig. 5 e).

Integrin  $\alpha 6 \beta 4$  plays a central role in the binding of hemidesmosomes to laminin-332 (laminin 5), which is an important constituent of the basement membrane on which basal epidermal keratinocytes are residing (Aumailley and Rousselle, 1999).

Integrin  $\alpha 6$  (Figs. 5d,e, f), and laminin-332 (laminin 5, Figs. 5g, h, i) as well as laminin 511 (laminin 10, data not shown), were found to be expressed in the basement membranes of KdES and SdES. For both proteins the expression pattern did not differ from that of normal skin (for comparison see suppl. Figs. S1b, g). However, in SdES, homogeneous expression was delayed.

K5 is expressed in the basal layer and the first suprabasal layers in normal human homeostatic epidermis (suppl. Fig. S1g). However, expression of K5 can be induced in migrating (activated) keratinocytes at wound edges (Patel et al., 2006). Our analyses revealed that in KdES almost the normal staining pattern was found after three weeks (Fig. 5g), whereas in SdES, K5 was strongly expressed suprabasally (Fig. 5h). Seven weeks after transplantation both types of epidermal substitutes showed a K5 distribution that was similar to that of normal human epidermis (Fig. 5i).

K15 is a useful indicator of epidermal homeostasis, as it is almost exclusively expressed in basal keratinocytes in a homeostatic epidermis (Pontiggia et al., 2009). Indeed, it is absent during wound healing (Porter et al., 2000; Waseem et al., 1999). Consistently, K15 was neither expressed in keratinocytes and SwGld cells grown on culture plastic, nor in KdES/SdES before transplantation. After transplantation K15 became up-regulated in the stratum basale which then exhibited the K15 expression pattern of normal human skin (Figs. 5k,l,m; compare to Fig. S1h).

K19 is considered a marker of very “young”, proliferative human skin (from children, younger than two years) (Pontiggia et al., 2009). In normal interfollicular epidermis, K19-positive keratinocytes are clustered in the stratum basale as a subpopulation of K15-positive basal keratinocytes in young skin and disappear with age (Pontiggia et al., 2009) and Fig. S1h. Figs. 5k and 5l show K19 expression in both KdESs and SdES three weeks after transplantation. Note that in Fig. 5l some K19-positive keratinocytes are still distributed suprabasally, indicating that tissue homeostasis was not yet completely established. In 7 weeks old transplants, K19 expression was already undetectable in both types of substitutes (Fig. 5m). The expression patterns of all markers employed in this study are summarized in Tab. 2.

## Ultrastructural analysis of the epidermal substitutes

Using transmission electron microscopy (TEM), we set out to study the two types of epidermal substitutes on an ultrastructural level. Fig. 6a shows an overview of a SdES, whereas the same structures in KdES are not shown. Quite frequently, blood vessels containing erythrocytes were evident (data not shown). The transplants were generally readily tolerated by the rats. Accordingly macrophages and leukocytes in the dermal extracellular matrix were rare. Relatively large epidermal intercellular spaces, indicative of active fluid transport and occasional autophagosomes in the epithelial cells (asterisk in Fig. 6b) denote a high metabolic turnover in the epidermal substitutes.

Fig. 6b illustrates a SwGld-derived cell with clearly visible intermediate filament bundles (white arrow). This basal cell formed hemidesmosomes (large black arrows) connecting to the basement membrane (group of small black arrows). The complex organisation of hemidesmosomes is illustrated in Fig. 6c. In the spinous layer, SwGld cells interconnected to one another via desmosomes (Fig. 6d). All structures indicative for a normal cornified envelope can be detected (Fig. 6e): In the upper spinous layer (ss) cells flattened during keratinization and became considerably smaller. Keratohyalin granules (Fig. 6e, arrows) were detected in the cells of the granular layer (sg). They increased in number and size towards the stratum granulosum, whereas the nuclei (nu) disappeared. Finally, an electron-dense stratum corneum, consisting of flattened cells devoid of organelles, as well as detaching squames (sq) were evident. Fig. 6f shows the magnification of a lamellar body in contact with the plasma membrane.

Taken together, TEM analyses of SdES revealed that human eccrine SwGld cells can form a stratified epidermal substitute which ultrastructurally is indistinguishable from a KdES.

## Discussion

A central issue of engineering human skin substitutes is the number and quality of epidermal keratinocyte stem cells present in the keratinocyte preparation. A distinct keratinocyte stem cell pool, referred to as the epidermal proliferative unit (EPU) is commonly thought to be located in the epidermal stratum basale (Ghazizadeh and Taichman, 2005; Kaur, 2006; Strachan and Ghadially, 2008). An additional source of self renewing keratinocytes is the hair follicle (Alonso and Fuchs, 2003; Blanpain and Fuchs, 2009; Cotsarelis, 2006). However, there is evidence that keratinocyte stem cells are only released from the hair follicle bulge if induced by epidermal wounding (Ito et al., 2005; Levy et al., 2005; Levy et al., 2007). The fact that there are regions of the human body that are devoid of hair follicles, such as palmo-plantar skin, indicate that hair follicles are not an exclusive source for keratinocyte stem cells.

An additional appendage of human skin is the eccrine sweat gland. Almost the entire human body is covered with sweat glands. One may therefore ask whether, in analogy to hair follicles, sweat glands can give rise to interfollicular keratinocytes in wound situations, or even serve as a permanent source of interfollicular keratinocyte stem cells (see also Fig. 7).

Employing a combined in vitro/in vivo bioassay, we show here that epidermal substitutes can be generated from sweat gland derived epithelial cells. These substitutes can be further differentiated and maintained in homeostasis on experimental animals for at least two months. They exhibit almost all the properties of an epidermis derived from *a priori* epidermal keratinocytes (with the exception of K2e expression, which appears to be retarded). This is substantiated by the following findings: (1) A stratified epidermis consisting of 10 -12 cell layers is formed by SwGld cells. (2) A distinct, anuclear stratum corneum develops and is maintained after

transplantation of SdES in vivo. (3) Proteins centrally involved in the process of cornification, such as filaggrin, loricrin, involucrin, envoplakin, periplakin, and the transglutaminases I and III, are expressed in a pattern that matches that of normal human skin. (4) Junctional complexes and hemidesmosomes are readily and regularly established. (5) K15 and K19-expressing keratinocytes become expressed in a distinct stratum basale three weeks after transplantation. K19 expressing cells disappear seven weeks after transplantation. (6) Cell proliferation in the basal layer reaches homeostatic levels. (7) The SwGld-derived epidermis is anchored within a well developed basal lamina. (8) Palmo-plantar or mucosal markers are not expressed in the SwGld-derived epidermis. (9) Like epidermal keratinocyte-derived substitutes, engineered SdES undergo maturation after their transplantation onto a living organism.

It was important to have our sweat gland preparations free of epidermal keratinocytes. This was reached by the micro-dissection technique, which was based on optical inspection of every single sweat gland, allowing the careful exclusion of the upper “epidermal” duct in 100% of the prepared sweat glands. The absence of epidermal tissue was confirmed by showing that K2e positive keratinocytes, constituting 80-90% of the cells of the human epidermis, were absent in the SwGld preparations. In addition we provided indirect indication that no cells of the epidermal stratum basale were “contaminating” the sweat gland cell fraction. Since there was no reliable antibody that allowed to discriminate between basal epidermal keratinocytes and sweat gland cells respectively, we did this by demonstrating that epidermal melanocytes were absent in the sweat gland cell fraction. Taken together, these lines of evidence strongly suggest that a pure SwGld cell population, consisting of secretory and ductal cells, has the potential to develop into a functional, stratified epidermis.



Although SdES exhibited almost the complete set of epidermal markers and all the feature of epidermal differentiation (see also Tab. 2) we realized a developmental delay in the expression of these markers and the establishment of epidermal homeostasis when compared to KdES. This is most likely due to SwGld cells undergoing a time consuming transition from ductal or secretory glandular cells to interfollicular epidermal keratinocytes. Thus, the delayed expression of markers like K1, K10, K5, K19, and occludin in SdES, is not really surprising. In addition, myoepithelial cells that were co-isolated with secretory SwGld cells may have a stabilizing effect on the SwGld cell phenotype in the organism. In our experimental setting, myoepithelial cells relatively rapidly disappeared in the cell cultures. Thus, the loss of myoepithelial cells, which are closely associated with secretory SwGld cells, in combination with the massive cell dissociation (representing an extreme wound situation) and the subsequent proliferation on cell culture plastic, may be the prerequisites for the transition of SwGld cells into epidermal keratinocytes.

SwGld cells are derived from ectodermal cells which differentiated into ductal and secretory glandular cells during embryogenesis (Fu et al., 2005). The cell transition described in this paper may represent the reversion of this ontogenetic process. There are two conceivable mechanisms:

- 1) A multipotent, SwGld-derived stem cell population proliferates and the resulting new cells differentiate in response to their new epidermal environment.
- 2) SG cells might de-differentiate into a more naive state, after which they differentiate into a new phenotype i.e. epidermal keratinocytes. Based on the fact that the new SG-derived epidermis reached a state of homeostasis, and was permanently maintained in vivo, it has to be postulated that self-renewing cells assured the integrity of the new epidermis. We consider it therefore likely that both, re-

programmed SG-derived cells and SG-derived stem cells in concert formed and maintained the new epidermis.

Interestingly, under no experimental circumstances, SwGld derived epithelial cells developed into SwGld-like structures. This suggests that the observed cell transition follows a default pathway of differentiation, which invariably causes SwGld cells to convert into epidermal keratinocytes. This pathway may well be of regulatory relevance. It is consistent with the concept that, in analogy to keratinocytes derived from human hair follicle bulge, a self renewing subpopulation of SwGld cells has the potential to reconstitute human epidermis upon wounding, or even to permanently participate in regenerating it (for an illustration see Fig. 7). As glabrous skin (such as palms and soles) is rich in SGs (Frinkel and Woodley, 2001), the presence of a self renewing, potentially epidermal cell population in SGs was repeatedly postulated (Barrandon, 2007; Brouard and Barrandon, 2003). The data presented in this paper, for the first time, provide evidence that significantly supports this assumption.

It remains to be investigated, which subtype of SwGld cells, secretory and/or ductal cells, is involved in epidermal regeneration and maintenance. Some recent evidence suggests the presence of a label retaining, multi-potent stem cell population in the secretory domain of the human eccrine sweat gland (Nakamura and Tokura, 2009). Apart from that, only few data can be found in the literature which support the ability of human SwGld cells to form an epithelium in culture and in vivo. In 1998 Miller et al. reported that in pigs the “sweat apparatus” can re-epithelialize skin ‘de novo’ after wounding (Miller et al., 1998). These authors concluded that porcine SwGld cells formed an epithelium that had morphological and structural features of palmo-plantar or buccal epithelia, rather than properties of trunk epidermis. However, in contrast to human skin which contains eccrine SwGlds, porcine skin harbors apocrine glands,

the function of which is different from that of human SwGlds (Ferry et al., 1995; Montagna and Yun, 1964).

In conclusion, this is the first study generating comprehensive evidence that a near normal epidermis can be cultured from sweat gland cells in vitro. Furthermore, the SdES can be successfully transplanted onto immuno-incompetent rats on which they adhere, further differentiate, and survive as a mature graft, strikingly similar to KdES. This study enlarges understanding of skin biology in that it corroborates the thesis that SwGld cells can switch their phenotype and become keratinocytes. From a tissue engineering point of view, SwGld-derived epithelial cells obviously represent an additional source of keratinocytes to grow a near normal autologous epidermis. This is particularly relevant for patients requiring large and urgent covering of skin defects, such as severe burn injuries (3rd degree; >50% BSA). In these cases self renewing keratinocytes are urgently required for the in vitro production of (as many as possible) life saving skin grafts.

## **Materials and Methods**

### **Preparation of skin cells**

Human skin samples from scalp, abdomen, retroauricular skin or foreskins were obtained from patients ranging in age between 1 and 18 years, after permission by the ethic commission of the Canton Zurich and after informed consent given by parents or patients. The skin samples were used for the isolation of SwGld cells, keratinocytes, and fibroblasts, or employed for histological analysis.

### **Establishment of primary cell cultures**

Keratinocytes and fibroblasts were isolated as described (Pontiggia et al., 2009). For SwGld cells, we used the following procedure:

The day before isolation mitomycin-treated or irradiated swiss albino 3T3 mouse fibroblasts (ATCC CCL-92) were seeded at a density of 10'000 per cm<sup>2</sup> in DMEM, 10% calf serum and 5 µg/ml gentamycin. Just before plating the fragments of sweat glands, the medium was changed to Rheinwald and Green medium (RGM) (Jones et al., 1988; Schon et al., 1999), i.e. 3 parts of DMEM and one part of Ham's F12, 10% FCS, 5 µg/ml gentamycin, 1.4 mM CaCl<sub>2</sub> (all Sigma, Buchs, Switzerland), 0.4 µg/ml hydrocortisone, 5 µg/ml insulin, 2 nM triiodothyronine, 180 µM adenine, 10 ng/ml EGF (all from Invitrogen, Basel, Switzerland) and 0.1 nM cholera toxin (Calbiochem/VWR International AG, Dietikon, Switzerland). Skin samples were cut into small pieces (~6 mm<sup>2</sup>), digested in DMEM containing 12 U/ml dispase (Invitrogen, Basel, Switzerland), 2 mg/ml collagenase blend F (Sigma, Buchs, Switzerland) and 38 U/ml collagenase II (Worthington, Bioconcept, Allschwil, Switzerland) for 16 h at 4°C and 4 h at 37°C until the mesenchyme was almost completely digested. The tissue pieces were centrifuged and fat droplets were

removed with the supernatant. The pellet was resuspended in 20 ml of DMEM containing 3-fold concentrated antibiotics (gentamycin 15 µg/ml, penicillin 3000 U/ml, streptomycin 3 mg/ml and fungizone 750 ng/ml), 1% FCS and transferred to a new culture dish. The dermal tissue was removed from the epidermis and fragments of sweat glands were collected employing a stereomicroscope and micropipettes. The fragments were washed with culture medium and finally transferred to the culture dish. At days 4-5 the first outgrowing cells were visible. After two weeks the feeder cells were removed by digestion with 0.1% Trypsin, 1 mM EDTA (Invitrogen, Basel, Switzerland) for 2 min at 37°C. The remaining SwGld cells were washed twice in PBS and detached from the dish with 0.5% trypsin, 5 mM EDTA, for 5 min at 37°C. Trypsin activity was stopped by the addition of 3.75 mg/ml soy bean trypsin inhibitor (Invitrogen, Basel, Switzerland). An almost single cell suspension was reached by repeated pipetting through a fine pipette tip.

Keratinocytes and SwGld cells were further expanded during passage 1. Keratinocytes were grown in a serum-free medium (SFM, Invitrogen, containing 0.2 ng/ml EGF and 25 µg/ml Bovine Pituitary Extract). SwGld cells were maintained in RGM.

### **Organotypic cultures**

Organotypic cultures were prepared using a previously established transwell system consisting of 6 well cell culture inserts with membranes of 3.0 µm pore-size (BD Falcon, Basel, Switzerland) (Pontiggia et al., 2009). The membranes were covered with collagen type I hydrogels containing human dermal fibroblasts (passage 1).

The collagen matrix was prepared according to the protocol of Costea et al. (Costea et al., 2003). Briefly, 0.7 ml of rat tail collagen type I (3.2-3.4 mg/ml, BD Biosciences, Allschwil Switzerland), were added to 0.2 ml chilled neutralization buffer containing

0.15 M NaOH and  $1 \times 10^5$  fibroblasts. After polymerization (10 min at room temperature and 20 min at 37°C) these dermal equivalents were grown in DMEM/10%FCS for 5 days. Subsequently, keratinocytes and SwGld cells were seeded onto each dermal equivalent at a density of  $125 \times 10^3$  cells per  $\text{cm}^2$  within siliconized polypropylene rings of 5 mm in diameter to avoid dispersion. After 7h the rings were removed, 1 ml RGM was added in the upper chamber, and 2 ml were added to the lower chamber. Triplicate wells were set up for each dermo-epidermal substitute. The constructs were cultured in RGM. After 4 days the dermo-epidermal substitutes were raised to the air/liquid interface and cultured for 3 additional weeks. The medium was changed every 2nd day. Cultures were finally processed for transplantation or for cryo- and paraffin sections.

### **Transplantation of cultured dermo-epidermal composites**

Dermo-epidermal grafts were transplanted onto full thickness skin defects created surgically and encased by polypropylene rings, 27 mm in diameter as previously described (Pontiggia et al., 2009). The rings were sutured on the back of 10 weeks old, female athymic Nu/Nu rats. The transplants were covered with a silicon foil. After 14 days the grafts were excised and processed for cryo- and paraffin sections. Anesthesia for all procedures was performed using isoflurane (Abbott AG, Baar, Switzerland).

### **Histology and Immuno-fluorescence**

The epidermal substitutes were embedded in OCT compound (Sakura Finetek/Digitana AG, Horgen, Switzerland) and frozen at -20°C.

Melanin was visualized using the Masson-Fontana technique with nuclear fast red as counterstain (Stevens and Chalk, 1996).

Cryosections were fixed and permeabilized in acetone for 5 min at -20°C, air dried, washed 3x in PBS and blocked in PBS, containing 2% BSA (Sigma, Buchs, Switzerland) for 30 minutes. Incubation with the diluted antibodies was performed in blocking buffer for 1 hour at room temperature. Slides were washed three times for 5 min in PBS and blocked for additional 15 min before the second antibody was added. If necessary the same procedure was repeated for the third antibody incubation. Finally the slides were incubated for 5 min in PBS containing 1 µg/ml Hoechst 33342 (Sigma, Buchs, Switzerland), washed twice for 5 min in PBS and mounted with Dako mounting solution (Dako, Baar, Switzerland) containing 25 mg/ml of DABCO anti-quenching agent (Sigma, Buchs, Switzerland).

To stain freshly isolated sweat glands cells the glands were dissociated for 3 min by means of concentrated (3x) trypsin/EDTA (Invitrogen, Basel, Switzerland). After inactivation of trypsin, the cells were fixed in 2% PFA for 10 min, permeabilized with 0.5% saponin for 5 min and incubated with the primary antibodies for 15 minutes on ice. The cells were passed through a 0.2 ml-serum layer by centrifugation and resuspended in PBS containing 2% BSA and the fluorescent-dye- conjugated secondary antibody for 15 min on ice. After a second serum-washing step the cells were centrifuged onto glass slides and the cytopins were analyzed by fluorescence microscopy.

## **Antibodies**

For IF the following antibodies were used: From Dako (Baar, Switzerland): K10 (clone DE-K10, 1:100), E-Cadherin (clone NCH-38, 1:30), laminin 10 (clone 4C7, 1:25), K19 (clone RCK108, 1:100), Melanosome (clone HMB45, 1:50); from Progen (Heidelberg, Germany): K2e (clone Ks 2.342.7.4, 1:100), K9 (polyclonal, 1:200), K5 (polyclonal, 1:100); from Chemicon (Millipore AG, Zug, Switzerland): K16 (clone

LL025, 1:100), K1 (clone LHK1, 1:200),  $\alpha$ 6 integrin (clone 4F10, 1:100), K15 (clone LHK15, 1:100); from Zymed (Invitrogen, Basel, Switzerland): occludin (polyclonal, 1:50), desmoglein 1 (clone 27B2, 1:50), desmoglein 3 (clone 5G11, 1:200); from LabVision (P.H.Stehelin&CIE AG, Basel, Switzerland): involucrin (clone SY5, 1:100), filaggrin (FLG01, 1:100); from Genetex (Biozol, Eching, Germany): K8 (clone B391, 1:200); from Sigma (Buchs, Switzerland): K4 (clone 6B10, 1:600); from BD Pharmingen (Basel, Switzerland): Ki-67 (clone B56, 1:200); from Abcam (Cambridge, UK): loricrin (polyclonal, 1:500); from Santa Cruz (Labforce AG, Nunningen, Switzerland): laminin 5 (clone P3H9-2, 1:100). The periplakin and envoplakin antibodies were a kind gift of Dr. Fiona Watt (Cambridge Research Institute, UK). As a secondary antibody we used FITC-conjugated polyclonal goat F(ab')<sub>2</sub> fragments directed to mouse immunoglobulins (Dako, Baar, Switzerland). For double IF, some of the primary antibodies were pre-labeled with Alexa 555-conjugated polyclonal goat F(ab')<sub>2</sub> fragments, according to the instructions of the manufacturer (Zenon Mouse IgG Labeling Kit, Molecular Probes, Invitrogen, Basel, Switzerland).

### **Fluorescence microscopy**

Fluorescence microscopy was performed using a Nikon Eclipse TE2000-U inverted microscope (Nikon AG, Egg, Switzerland) equipped with Hoechst, FITC, and TRITC filter sets. For confocal imaging we used a Leica SP1 confocal laser scanning microscope (Leica, Heerbrugg, Switzerland) equipped with Argon UV laser with 351 nm / 364 nm excitation, Argon/Krypton laser with 474 nm / 488 nm / 568 nm / 647 nm excitation, fluorescence filters for DAPI, FITC and TRITC. The line average was set to 4. Images were processed with Imaris 5.0.1 (Bitplane AG, Zurich, Switzerland).



## **Electron Microscopy**

For transmission electron microscopy (TEM) analysis, tissue blocs (approximately 1 mm<sup>3</sup>) were prefixed in 0.1 M cacodylate buffer (Merck, Hohenbrunn, Germany), pH 7.3 containing 2.5% glutaraldehyde for 2 hours, washed in cacodylate buffer, postfixed with an aqueous solution of 1% OsO<sub>4</sub> and 1.5% K<sub>4</sub>Fe(CN)<sub>6</sub> for 1 hours, dehydrated and finally embedded in EPON 812 (Catalys AG, Wallisellen, Switzerland). Ultrathin sections (approximately 50-70 nm) were collected on copper grids were contrasted with 4% uranyl acetate and 3% lead citrate, and examined with a CM 100 transmission electron microscope (Philips, Eindhoven, The Netherlands). All reagents were from Sigma (Buchs, Switzerland) unless mentioned otherwise.

## **In situ Hybridisation with Alu-probes**

The epidermal substitutes (ES) were fixed in 4% neutral formalin and embedded in paraffin according to standard protocols. We used basically the method published by Just et al. (Just et al., 2003). Briefly: The genomic DNA (gDNA) was extracted with the QIAamp DNA Blood Mini Kit (Qiagen) from the whole blood of a volunteer which donated 2 ml blood after written informed consent. 50 ng of gDNA and 0.5 µM of the primers (Alu-sense: 5' ACG CCT GTA ATC CCA GCA CTT 3'; Alu-antisense: 5' TCG CCC AGG CTG GAG TGC A 3' produced by Microsynth, Balgach, Switzerland) were applied in a PCR with Hot Star Taq DNA polymerase (Qiagen, Hombrechtikon, Switzerland) under the following conditions: 95°C for 15 min, 25x (94°C for 30 s, 58°C for 45 s, 72°C for 45 s) and 72°C for 10 min. The 245 bp-product was purified by agarose gel electrophoresis and the QIAquick Gel Extraction Kit (Qiagen). 200 pg were used as template for the DIG-labeling PCR (PCR DIG probe synthesis kit, Roche, Rotkreuz, Switzerland) under the same conditions but with increased MgCl<sub>2</sub> concentration (5 mM). The DIG-labelled probe was purified by ethanol precipitation.

Paraffin sections were mounted on superfrost slides, deparaffinized (xylol, 3x 10 min), hydrated (ethanol 100-96-80-70-50%, 2 min each) and washed in PBS (3x 5 min). Cryosections (16 µm thick) were mounted on superfrost slides, dried for 30 min at 37°C, washed in PBS (3x 5 min), fixed with 4% PFA for 20 min and washed again. Both section types were further permeabilized (0.3% Triton X-100 in PBS, 10 min), digested with proteinase K (2 µg/ml in 100 mM Tris/HCl pH 8, 50 mM EDTA, 15 min at 37°C, cryosections 5 min only), acetylated (0.1 M triethanolamine, 0.25% Acetic anhydride, 2x 5 min) and washed first in PBS (2x 5min) and finally in 2xSSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0, 5 min). The hybridisation was performed in a moist chamber with 50% formamide. The slides were incubated in hybridization buffer (50% formamide in 5x SSC, 0.15% N-lauroylsarcosine, 0.02% SDS, 2% blocking reagent) without probe for 30 min at 85°C and with 100 ng/ml DIG-labelled DNA-Probe for 30 min at 85°C. After chilling on ice for 10 min the slides were incubated over night at 42°C and finally washed in 2x SSC (briefly) and 0.1x SSC (3x 15 min at 42°C). For staining the sections were blocked (30 min in 0.15 M NaCl, 0.1 M maleic acid pH 7.5, 1% blocking reagent), incubated with an alkaline phosphatase-conjugated anti-DIG antibody (1:2000 in blocking buffer and 0.1% Triton X-100, 60 min), washed (4x 15 min in 0.15 M NaCl, 0.1 M maleic acid pH 7.5), equilibrated in TBS (0.1 M Tris pH 9.5, 0.1 M NaCl, 5 min), incubated with the substrate solution (200 µl of Nitroblue tetrazolium salt + bromo-chloro-indolyl phosphate in 10 ml TBS, 120 min) and the stop solution (10 mM Tris pH 8.0, 1 mM EDTA, 2 min) and washed in PBS (3x 15 min, second wash containing 1 µg/ml Hoechst 33342). Finally the slides were mounted in Eukitt® quick-hardening mounting medium. All reagents were from Fluka (Buchs, Switzerland); the proteinase K and the DIG nucleic acid detection kit including blocking reagent and substrate solution were from Roche (Rotkreuz, Switzerland).

## **Conflict of interest**

The authors state no conflict of interest.

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**Table 1. The expression patterns of epithelial markers**

Marker	Antibody	Human epidermis		Human sweat gland		
		Suprabasal KC	basal KC	Acro-syringium	duct	secretory domain
CEA	Polyclonal	-	-	-	+	+
K1	LHK1	+	-	+	-	-
K5	Polyclonal	(+)	+	-	+	+
K8	B391	-	-	-	-	+
K10	DE-K10	+	-	+	+	-
K14	LL002	(+)	+	-	+	+
K16	LL025	+ *	+ *	n.d.	+	(+)
K19	RCK108	-	+ °	+	+	+
Involucrin	SY5	+	-	+	+	-
K1b (K77)	Polyclonal	+	-	-	+	-
K2e	Ks 2.342.7.4	+	-	-	-	-
K15	LHK15, SPM190	-	+	-	-	+
K17	Ks 17.E3	-	-	-	+	+
K20	Ks 20.8	-	-	-	-	-
Desmoglein 1	27B2	+	-	n.d.	-	-
Desmoglein 3	5G11	+	+	n.d.	+	-
Connexin 26	CX-1E8	-	-	-	+	(+)
Integrin $\alpha 6$	4F10	-	+	-	+	+
Aquaporin 5	Polyclonal	-	+	n.d.	-	+
Follistatin	85918	-	+	-	-	+
CD200	MRC OX104	-	-	-	+	-
Na,K-ATPase $\alpha 1$	Polyclonal	+	+	n.d.	+	+
Transglut. 1	Polyclonal	+	-	n.d.	-	-
Transglut. 3	Polyclonal	+	-	n.d.	-	-
Transglut. 5	Polyclonal	+	+	n.d.	+	+

\* Wound healing marker  
 ° new-born  
 n.d. not determined  
 + expressed  
 - not expressed  
 (+) minimal expression  
 KC keratinocytes



**Table 2. Expression of epidermal markers in KdES and SdES**

Antigen	Antibody (Clone)	Normal Skin	KdES 3 weeks	SdES 3 weeks	SdES 7 weeks	
Occludin	polyclonal	●●	●●	●	●●	
Loricrin	polyclonal	●●	●	●	●	
Involucrin	SY5	●●	●	●	●	
Periplakin	polyclonal	●●	●●	●●	●●	
K1	LHK1	●●	●●	●	●●	
K10	DE-K10	●●	●●	●●	●●	
K2e	Ks 2.342.7.4	●●	●●	(●)	(●)	
K16	LL025	-	●●	●●	●●	
K9	polyclonal	-	-	-	-	only in palmoplantar skin
Desmoglein 1	27B2	●●	●	●	●	
Desmoglein 3	5G11	●●	●	●	●	
E-Cadherin	NCH-38	●●	●●	●●	●●	
K5	polyclonal	●●	●	●	●	
K15	LHK15, SPM190	●●	●●	●●	●●	
K19	RCK108	●●	●●	●●	-	
Integrin α6	4F10	●●	●●	●●	●●	
Laminin 5	P3H9-2	●●	●●	●●	●●	
Laminin 10	4C7	●●	●●	●●	●●	
Ki-67	B56	●●	●	●	●	
K8	B391	-	-	-	-	only in secretory SG
K4	6B10	-	-	-	-	only in mucosal epithelium

●● normal expression

● reduced expression

(●) expressed in single scattered cells

- no expression

KdES: keratinocyte derived epidermal substitute

SdES: sweat gland derived epidermal substitute

## Figure Legends

### **Figure 1. Macroscopic and histologic appearance of SdES and KdES *in vitro* and after transplantation onto immuno-incompetent rats.**

(a) Isolation of human sweat glands after dispase/collagenase digestion. Sweat gland fragments containing the glandular domain (white arrowheads) and parts of the intradermal ducts (black arrows). (b) A typical epithelial cell colony growing out from a human sweat gland fragment. (c, d) Keratinocytes and sweat gland cells grown at the air/liquid phase on collagen type I hydrogels on permeable inserts. Keratinocytes were initially seeded within a ring area of 0.7 cm<sup>2</sup>. Three weeks after plating this area was increased by about 4 times. Sweat gland cells increased this area by approx. 2.5 times. (e, f) KdES in e, and SdES in f, three weeks after transplantation onto full thickness skin wounds on the back of athymic rats. (g,h) Hematoxylin/eosin staining of KdES (g) and SdES (h) three weeks after transplantation reveals a stratified multilayer consisting of a basal layer, 10-12 suprabasal layers and a stratum corneum in both types of substitutes. Scale bars in a) 500 µm, in b) 200 µm, in g, h) 100 µm. The rings in e) and f) are 2.7 cm in diameter.

### **Figure 2. Tissue specific markers in KdES and SdES.**

Double immuno-fluorescence using the antibody combinations K2e/K8 (a, b, c, d, e, f) and K9/K4: (g, h). (a) K2e is expressed in suprabasal layers (white arrowhead), as demonstrated in the area of the glandular acrosyringium. Note that the SwGld duct (dotted circles), meandering through the acrosyringium, does not express K2e. K8 is not expressed in normal human epidermis. (b) In normal human skin the duct (du) of an eccrine sweat gland is negative for K8 (and K2e), whereas the secretory domain

(se) expresses K8. (c) Cytospin of a freshly isolated human sweat gland cell suspension. About 50% of the cells are positive for K8. All SwGld cells are negative for K2e. (d) In contrast, freshly isolated human keratinocytes are positive for K2e and negative for K8. (e) Immuno-fluorescence staining for the expression of K2e and K8 of KdES three weeks after transplantation. (f) Only a few single cells are staining for K2e/K8 in SdES. (g, h) Almost no expression of the palmoplantar marker K9 and the mucosal marker K4 in KdES (g) and SdES (h) after transplantation. (i) In-situ hybridization of SdES using a DNA-probe that hybridizes to human-specific Alu-sequences. Positive (violet) staining proves the human origin of the dermo-epidermal graft. The boundary between human tissue and the underlying unstained rat tissue is obvious. White dotted line: Dermo-epidermal border. Scale bars for all panels: 50  $\mu$ m.

**Figure 3. Preparations of human eccrine sweat gland epithelial cells are not contaminated by basal epidermal cells.**

a) Histological section of a typical dermo-epidermal substitute (KdES) transplanted onto immuno-incompetent nu/nu rats 3 weeks after transplantation. Melanocytes (HMB45, green cells) located in the stratum basale are obvious. The basement membrane is stained by a laminin 332 antibody. b) No melanocytes are detectable in sections of SdES, indicating that no basal epidermal cells, hence no basal epidermal keratinocytes, were present in the initial sweat gland cell preparation. c) Fontana-Masson staining reveals the melanin content both in basal melanocytes and epidermal keratinocytes. d) No melanosomes are detectable in SdES. Bar for a), b), c), d). e) Magnified detail of c). f) Magnified detail of d). Bar for a-f) 50  $\mu$ m.

**Figure 4. Epidermal stratification and homeostasis in KdES and SdES after transplantation onto immuno-incompetent rats.**

Double immuno-fluorescence using antibodies to Ki-67/Cytokeratin 16 (a,b,c), Loricrin/Involucrin (d,e,f), Cytokeratin 1/Cytokeratin 10 (g,h,i), Occludin/E-cadherin (k,l,m). **(a, d, g, k)** Indirect immuno-fluorescence reveals the usual epidermal differentiation of KdES three weeks after transplantation. **(b, e, h, l)** Epidermal differentiation of SdES three weeks after transplantation. A delayed differentiation is obvious with respect to the expression pattern of K1/K10 (h). **(c, f, i, m)** Epidermal differentiation of SdES seven weeks after transplantation. No difference to the control situation (compare to a, d, g, k) can be observed. White dotted line: Dermo-epidermal border. Scale bar for all panels: 50  $\mu$ m.

**Figure 5. Epidermal stratification and homeostasis in KdES and SdES after transplantation onto immuno-incompetent rats.**

Double immuno-fluorescence of cryosections using antibodies to: Desmoglein1/Desmoglein3 (a,b,c), Periplakin/Integrin- $\alpha$ 6 (d,e,f), Cytokeratin 5/Laminin 5 (Laminin-332) (g,h,i), Cytokeratin 15/Cytokeratin 19 (k,l,m). **(a, d, g, k)** Indirect immuno-fluorescence displays the typical epidermal differentiation of KdES three weeks after transplantation. **(b, e, h, l)** Epidermal differentiation of SdES three weeks after transplantation. A delayed differentiation is visible with respect to the expression pattern of Dsg1/3 (b), integrin  $\alpha$ 6 (e) and K5 (g). Note that some K19-positive keratinocytes are still distributed suprabasally (white arrows in l). **(c, f, i, m)** Epidermal differentiation of SdES seven weeks after transplantation. No difference to the control situation (compare to a, d, g, k) can be observed. K19-positive keratinocytes in the stratum basale have already disappeared indicating advanced

tissue homeostasis and differentiation. White dotted line: Dermo-epidermal border.  
Scale bar for all panels: 50  $\mu\text{m}$ .

**Figure 6. Transmission electron microscopy of a SdES after transplantation reveals that SwGld-derived cells develop into characteristic human epidermal structures.**

(a) The typical epidermal stratification and the characteristic adhesion of epidermal keratinocytes by numerous desmosomes are obvious. The lower pale cells are dermal fibroblasts (F). Dark cells are keratinocytes (K). (b) A mature dermo-epidermal junction has developed. A basal lamina (group of small arrows) and hemidesmosomes (black filled arrows) as well as the keratin filament network (white single arrow) and an autophagosome (asterisk) in an epithelial cell are indicated. (dem = dermal extracellular matrix, F = fibroblast) (c) The complex structure of a hemidesmosome is shown (pm = plasma membrane, ip = inner plate, op = outer plate). A complete basal lamina is deposited consisting of the sublamina dense plate (white arrow), the lamina lucida (black arrow) and the lamina densa (ld). (d) Mature desmosomal connections (black arrows) between adjacent epithelial cells of the spinous layer. The inset depicts a typical desmosome at a higher magnification. (e) Magnification of the upper epidermal layers showing the stratum spinosum (ss), the stratum granulosum (sg) containing the dark keratohyalin granula (white arrows), the stratum corneum (sc) and skin squames (sq). (f) A lamellar body (lb) between two corneocytes in the stratum corneum (sc) is depicted. Scale bars: 10  $\mu\text{m}$  (a, e), 5  $\mu\text{m}$  (b), 1  $\mu\text{m}$  (d) and 0.2  $\mu\text{m}$  (c, f).

**Figure 7. Hypothetical scheme considering both, the human eccrine sweat gland and the human hair follicle as sources for epidermal keratinocyte (stem) cells.**

The human hair follicle bulge (bg) gives rise to epidermal keratinocytes that migrate towards the epidermis as it is locally injured upon wounding (ch = club hair). As eccrine SwGld cells have the general potential to generate an epidermis (ep), SwGld cells derived from the secretory domain (sd) or ductal domain (dd), may also migrate towards the epidermal stratum basale (sb) to participate in closing an epidermal wound. It may even be considered that SwGld cells are involved in the homeostatic maintenance of the human epidermis.

**Figure S1. Distinct markers in normal human skin.**

Indirect immuno-fluorescence of normal human skin using antibodies to (a) Ki-67/Cytokeratin 16, (b) Periplakin/Integrin- $\alpha$ 6, (c) Loricrin/Involucrin (d) Cytokeratin 1/Cytokeratin 10, (e) Occludin/E-cadherin, (f) Desmoglein1/Desmoglein3, (g) Cytokeratin 5/Laminin 5 (Laminin-332) and (h) Cytokeratin 15/Cytokeratin 19. These antibodies were employed to create a normal reference for the analysis of our in vitro and vivo skin substitutes. Scale bar for all panels: 50  $\mu$ m.